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EVIDENCE FOR HIGH-AFFINITY Ca^{2+} -ATPase
ACTIVITY AND ATP-DRIVEN Ca^{2+} -TRANSPORT IN
MEMBRANE PREPARATIONS OF THE GILL
EPITHELIUM OF THE CICHLID FISH *OREOCHROMIS*
MOSSAMBICUS

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SUMMARY

A high-affinity Ca^{2+} -ATPase activity was demonstrated among the phosphatase activities in plasma membranes of tilapia branchial epithelium; its characteristics ($K_{0.5} = 0.063 \mu\text{mol l}^{-1} \text{Ca}^{2+}$, $V_{\text{max}} = 6.02 \mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$ protein at 37°C) resemble those of Ca^{2+} -translocating enzymes present in mammalian erythrocytes or enterocytes. The ratio of this Ca^{2+} -ATPase activity to Na^+/K^+ -ATPase activity was 1:20.4. Radioimmunoassayable calmodulin was demonstrated in the Ca^{2+} -ATPase-containing membrane fraction.

ATP-dependent Ca^{2+} -transport was demonstrated in tight-vesicle preparations of the branchial cell membranes; 30 % of the vesicles in the preparation were inside-out, 44 % were right-side-out and 26 % were leaky. The characteristics of the active Ca^{2+} -transport activity are consistent with a Ca^{2+} -extrusion mechanism involving high-affinity Ca^{2+} -ATPase activity.

The branchial Ca^{2+} -transport activity per fish, as calculated on the basis of the transport activity determined for the vesicle preparation, is of the order of the branchial Ca^{2+} -influx rates observed *in vivo*. The data provide the first biochemical evidence for active Ca^{2+} -transport in plasma membranes of branchial epithelium. A model is presented for the mechanism of active transepithelial Ca^{2+} -transport in fish gills.

INTRODUCTION

For a fish to live and grow in fresh waters, which are generally hypocalcic to the body fluids, a permanent need exists to establish a positive calcium balance. Gills are of major importance for the uptake of Ca^{2+} (Simmons, 1971; So & Fenwick, 1977; Payan, Mayer-Gostan & Pang, 1981). When fish are fed a calcium-deficient diet, growth is not hampered provided the calcium level of the water is not too low (Rodgers, 1984). Little is known about the mechanisms involved in Ca^{2+} -transport in fish

Key words: Freshwater teleost fish, gills, high-affinity Ca^{2+} -ATPase, ATP-driven Ca^{2+} -transport.

gills. Several authors have described the presence in gills of Ca^{2+} -activated ATPase activity, presumed to be the enzymatic basis for active transport of Ca^{2+} through the gills. However, we have shown for the gills of American eels (*Anguilla rostrata*) that the Ca^{2+} -activated ATP-hydrolysis previously described results from the activity of a heterogeneous pool of phosphatases present in the plasma membranes of the gill epithelium (Flik, Wendelaar Bonga & Fenwick, 1983). We also presented evidence for the presence of a high-affinity Ca^{2+} -ATPase activity in the eel gill plasma membranes (Flik *et al.* 1984b,c). Characteristics of the latter enzymic activity are: an affinity for Ca^{2+} ($0.22 \mu\text{mol l}^{-1} \text{Ca}^{2+}$) in the intracellular Ca^{2+} -concentration range, ATP-preference, and calmodulin-dependency. These characteristics are shared with established mammalian Ca^{2+} -ATPases involved in Ca^{2+} translocation over plasma membranes (Vincenzi & Larsen, 1980; De Jonge, Ghijsen & Van Os, 1981; Ghijsen, De Jong & Van Os, 1982). Induction of high-affinity Ca^{2+} -ATPase activity in eel gill plasma membranes was observed after treatment of eels with the hypercalcaemic hormone prolactin, which further indicated an involvement of this enzyme in the active uptake of Ca^{2+} from the water (Flik *et al.* 1984c). To our knowledge, these are the only reports so far of a Ca^{2+} -ATPase activity in fish that fulfils criteria for an enzyme to be directly involved in transcellular Ca^{2+} -transport.

The present study was undertaken to assess whether plasma membranes of the gills of the cichlid teleost tilapia (*Oreochromis mossambicus*) contain a high-affinity Ca^{2+} -ATPase activity and a Ca^{2+} -transport activity. Ca^{2+} -induced $\text{Mg}\sim\text{ATP}$ hydrolysis was studied in Na^+/K^+ -ATPase-enriched membrane fractions isolated from branchial epithelium. ATP-dependent Ca^{2+} -transport activity was determined in tight-vesicle preparations of these membranes. As a first step to verify whether these Ca^{2+} -dependent processes are calmodulin-dependent (as we have shown before for the high-affinity Ca^{2+} -ATPase activity in eel gills; Flik *et al.* 1984b), the presence and concentration of calmodulin in the gill membrane fraction were investigated. Membrane orientation in resealed vesicles was determined on the basis of the activity of acetylcholine esterase and glyceraldehyde-3-phosphate dehydrogenase. The total branchial Ca^{2+} -transport capacity *in vitro* was calculated and compared to branchial Ca^{2+} -influx rates as determined *in vivo*.

MATERIALS AND METHODS

Male tilapia, *Oreochromis mossambicus* (formerly *Sarotherodon mossambicus*), were used in all experiments. Body weights ranged from 10 to 30 g. Freshwater specimens were obtained from laboratory stock kept at 28°C as described previously (Wendelaar Bonga & Van der Meij, 1980). The Ca^{2+} concentration of the water was 0.8 mmol l^{-1} .

Reagent grade chemicals were purchased from Sigma. Ultrapure water was used in enzyme assays and in Ca^{2+} -transport studies that involved the use of Ca^{2+} -buffers.

Isolation of plasma membranes

To collect branchial epithelium, fish were anaesthetized in Tris-buffered MS-222 and, after transection of the spinal cord, the branchial apparatus was removed and the

epithelium scraped off onto an ice-cold glass plate. Scrapings were collected in an isotonic buffer containing (in mmol l⁻¹): sorbitol, 250; NaCl, 12.5; imidazol/histidine, 5.0, pH 7.5; phenylmethylsulphonylfluoride (PMSF), 0.2; dithiothreitol, 0.1; aprotinin, 100 U ml⁻¹ and EDTA, 0.1. Use of this buffer and a loosely-fitting dounce homogenizer (Braun Melsungen) allowed disruption of the branchial epithelium but left the erythrocytes intact. The latter were separated from the branchial epithelial membrane fragments by a 550 g, 10-min centrifuge run. Light-microscope examination of the pellet obtained after this centrifugation step revealed a homogeneous population of intact red blood cells and nuclei. Membranes of the disrupted epithelium were collected by centrifugation of the supernatant (designated H₀) remaining after separation of the erythrocytes, yielding a pellet (P₀) and a supernatant which was designated as the cytosolic fraction (S₀). P₀ was further purified by differential centrifugation as described for eel gill epithelial membranes (Flik *et al.* 1983), yielding a plasma membrane fraction called P₃ (method I), or by isopycnic centrifugation on a discontinuous sorbitol gradient, yielding a plasma membrane fraction called P₃' (method II). For gradient centrifugation P₀ was resuspended (100 strokes) with a douncer in 21 % sorbitol in homogenization buffer; a 3-ml aliquot of this suspension was layered on two blocks of 4.5 ml of 43 % and 36 % sorbitol and run at 200 K_g for 180 min at 4 °C (Beckman L8-80, SW40 Ti). The band appearing at the 43 % / 36 % sorbitol interface was collected and pelleted after dilution with sorbitol-free buffer by ultracentrifugation (300 K_g for 30 min; SW50 Ti). It proved to consist of membranes highly enriched in Na⁺/K⁺-ATPase activity. Method II was introduced to improve the detectability of high-affinity Ca²⁺-ATPase activity, which typically amounts to only 5 % of the Na⁺/K⁺-ATPase activity in plasma membrane preparations (De Jonge *et al.* 1981; Flik *et al.* 1984b).

Plasma membranes were resuspended in the basic assay buffer, divided in portions as required for the assays, frozen in liquid nitrogen and stored at -80 °C for a maximum of 1 week. For the preparation of sealed plasma membrane vesicles, EDTA was omitted after the initial homogenization step. Membranes to be used for Ca²⁺-transport studies were resealed in a buffer containing (in mmol l⁻¹): KCl, 150; MgCl₂, 5 and HEPES/Tris, 30; pH 7.4, according to the method of Ghijsen *et al.* (1982). Membranes isolated for orientation and Ca²⁺-transport studies were used immediately after isolation without being frozen.

Assays and assay media

Protein, Na⁺/K⁺-ATPase, succinic acid dehydrogenase (SDH) and Ca²⁺-stimulated ATPase activities were determined as described in detail elsewhere (Flik *et al.* 1983, 1984b). Calmodulin was determined using a commercial ¹²⁵I-radioimmunoassay kit (New England Nuclear, NEK-18) with a highly specific antibody raised in sheep against non-derivatized rat testis calmodulin. Membrane fractions (H₀, P₃') were assayed without further purification. The cytosolic fraction (S₀) was partially purified as suggested by Teo, Wang & Wang (1973) and as described in detail for the purification of calmodulin from fish mucus (Flik, van Rijs & Wendelaar Bonga, 1984a).

Membrane impermeability of resealed plasma membrane vesicles was tested by determining the effect of detergents on the activity of membrane-bound enzymes

Table 1. *Percentage recovery and specific activities of marker enzymes in tilapia gill membrane fractions*

	H ₀		P ₃		P ₃ '	
	% Recovery	V _{spec}	% Recovery	V _{spec}	% Recovery	V _{spec}
Protein	100	—	2.82 ± 0.34	—	0.36 ± 0.14	—
Na ⁺ /K ⁺ -ATPase	100	8.1 ± 2.9	4.52 ± 0.91	30.9 ± 1.9	4.38 ± 1.77	123.3 ± 33.0
SDH	100	—	1.21 ± 0.23	—	< 1	—

Recoveries are expressed as percentages of the total enzyme activities in the initial homogenate (H₀).

Na⁺/K⁺-ATPase specific activities (V_{spec}) are expressed in $\mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$ protein at 37°C.

Mean values ± S.E. are given; N = 5.

with established sidedness. Acetylcholine esterase (exoenzyme) and glyceraldehyde-3-phosphate dehydrogenase (GPDH, endoenzyme) activities were determined in resealed vesicle preparations treated with 0–0.2 % v/v Triton X-100, as suggested for erythrocyte membranes by Steck & Kant (1974). No modifications were introduced in these assays. Initial enzyme velocities (upon addition of substrate) in the presence of optimal detergent concentrations were equated with 100 % accessibility. Enzyme activities in the absence of detergent were expressed as percentages of the maximum activities observed. From these accessibility percentages for the two enzymes we calculated the percentage of inside-out (IOV), right-side-out (ROV) and leaky vesicles of a membrane preparation.

Ca²⁺-transport activity in gill plasma membranes was determined as ATP-dependent ⁴⁵Ca-accumulation over a 2- to 5-min period at 37 °C in resealed vesicles, according to the method of Ghijsen *et al.* (1982). The amount of protein per filter was 7.4 μg BSA equivalents.

Statistics and calculations

Values are expressed as means ± standard deviations. Statistical analysis of differences between mean values was carried out applying Student's *t*-test for unpaired observations ($\alpha = 5\%$). Significance was accepted at the 2 % level. Determination of $K_{0.5}$ for Ca²⁺ and V_{max} values from Eadie-Hofstee plots of Ca²⁺-stimulated ATPase and transport activities in plasma membranes was performed according to Borst Pauwels (1973). Linear regression analysis was based on the least-squares method.

RESULTS

Plasma membrane isolation

In Table 1 the percentage recovery of protein, Na⁺/K⁺-ATPase and SDH activities are presented for the isolation of plasma membrane enriched fractions (P₃ and P₃'). The P₃-fraction contained 2.82 % of the initial protein, 4.52 % of the initial Na⁺/K⁺-ATPase (used as plasma membrane marker) and 1.21 % of the initial SDH activity. In those cases, where P₃'-fractions were prepared, 0.36 % of the initial membrane protein, 4.38 % of the initial Na⁺/K⁺-ATPase and less than 1 % of the initial SDH activities were recovered. During these procedures the Na⁺/K⁺-ATPase

specific activities for the P₃- and P₃'-fractions increased approximately 3.5 and 12 times with respect to H₀, respectively. To exclude mitochondrial ATPase activity in assays for Ca²⁺-stimulated ATPase activities of plasma membrane, we routinely added oligomycin (5 µg ml⁻¹) and sodium azide (5 mmol l⁻¹) to the pertinent assay media.

In Table 2, Na⁺/K⁺-ATPase specific activities are given for P₃-fractions isolated in the presence or absence of EDTA. Na⁺/K⁺-ATPase specific activities for P₃-fractions isolated in the presence of EDTA amounted to 30.9 ± 1.9 µmol P_i h⁻¹ mg⁻¹ protein at 37 °C and were not affected by detergent treatment. Na⁺/K⁺-ATPase activities in membranes isolated in the absence of EDTA increased by a factor of 2.2 when pretreated with 0.1 % v/v Triton X-100 for 1 min at 37 °C, and reached levels similar to those observed in membranes isolated in the presence of EDTA. The activation of the extrinsic Na⁺/K⁺-ATPase indicates that isolation of membranes in the absence of EDTA yields a preparation that at least partially consists of resealed vesicles. Such resealed plasma membrane vesicle preparations showed 70 % acetylcholine esterase accessibility and 56 % GPDH accessibility. This indicated that the preparation consisted of 26 % leaky, 30 % inside-out and 44 % right-side-out vesicles. Consistent with these results was the observation, by transmission electron microscopy, of a homogeneous population of circular bilayer membranes with only a low incidence of membrane fragments (results not shown).

Ca²⁺-stimulated ATPase activity

In Fig. 1, Ca²⁺-stimulated ATPase activities determined in P₃'-fractions are shown. Ca²⁺-induced Mg~ATP-hydrolysis represented by an Eadie-Hofstee plot revealed a high-affinity and a low-affinity ATPase activity. *K*_{0.5} and *V*_{max} values for the high-affinity component were calculated as 0.063 µmol l⁻¹ Ca²⁺ and 4.9 % stimulation of the Mg~ATP-hydrolysis observed in the absence of Ca²⁺; for the low-affinity component these values were 14 µmol l⁻¹ Ca²⁺ and 10.7 % stimulation, respectively. The kinetic parameters for the high-affinity component resemble closely those reported earlier for the high-affinity Ca²⁺-ATPase of eel gill epithelium (Flik *et al.* 1984b). *V*_{max} for tilapia high-affinity Ca²⁺-ATPase in P₃'-fractions amounted to 6.02 µmol P_i h⁻¹ mg⁻¹ protein at 37 °C. The ratio of the Na⁺/K⁺-ATPase and the high-affinity Ca²⁺-ATPase in these membranes was 20.4 : 1, a value identical to the one reported for eel gill plasma membranes.

Table 2. Vesicle tightness and membrane orientation

	<i>N</i>	Isolated + EDTA		Isolated - EDTA	
		Triton X-100	Controls	Triton X-100	Controls
Acetylcholine esterase	8	—	—	100	70 ± 14
GPDH	8	—	—	100	56 ± 11
Na ⁺ /K ⁺ ATPase	5	100 (31.2 ± 2.4)	99 (30.9 ± 1.9)	100 (27.9 ± 5.7)	45 (12.5 ± 3.6)

P₃-fractions isolated in the presence or absence of EDTA were analysed for Na⁺/K⁺-ATPase, acetylcholine esterase and GPDH activity after treatment with buffered Triton X-100 (0.1–0.2 % v/v) or buffer only (controls). Enzyme activities determined in the presence of optimal concentrations of the detergent were designated 100 %.

Values for Na⁺/K⁺-ATPase specific activities (expressed in µmol P_i h⁻¹ mg⁻¹ protein, determined at 37 °C) are given in parentheses.

Mean values ± s.e. are given; *N* = number of determinations.

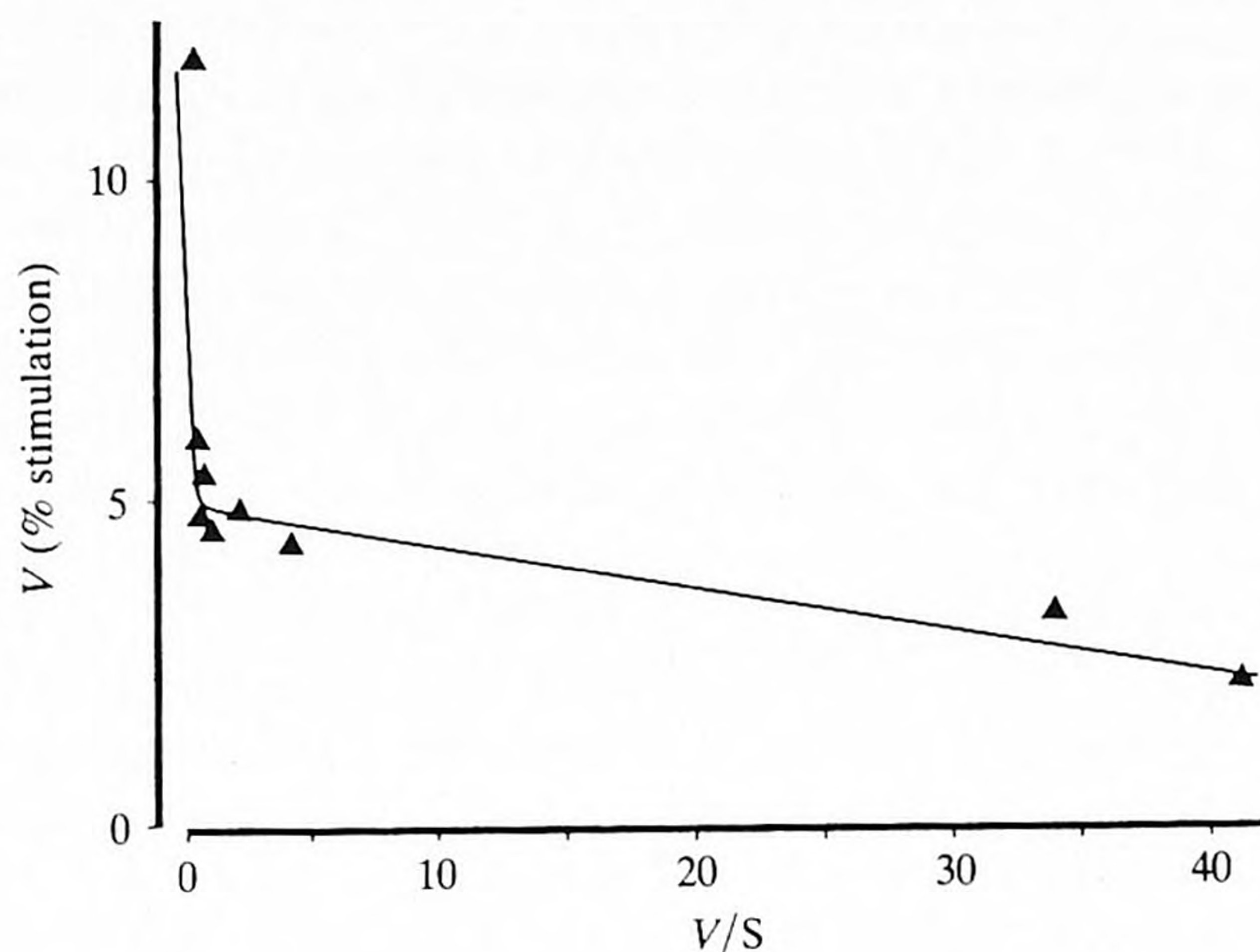


Fig. 1. Eadie-Hofstee plot of Ca^{2+} -stimulated $\text{Mg}\sim\text{ATP}$ -hydrolysis by plasma membranes of tilapia branchial epithelium. Enzymatic activities (V) were expressed as percentage stimulation above background hydrolytic activity. Ca^{2+} concentrations (S) are expressed in $\mu\text{mol l}^{-1}$.

Table 3. *Calmodulin in tilapia branchial epithelium*

Fractions	Calmodulin (mg g^{-1} protein)
H_0	1.65 ± 0.05
S_0	1.98 ± 0.12
P_3'	0.29 ± 0.03

Calmodulin concentrations were determined by radioimmunoassay.

H_0 refers to homogenate of branchial epithelium; S_0 refers to the supernatant obtained after ultracentrifugation of H_0 , and represents the cytosolic fraction of the branchial epithelium. S_0 was partially purified, as described in Materials and Methods.

P_3' is the membrane fraction obtained after gradient centrifugation; $N = 2$.

Presence of calmodulin

In our reports on eel gill plasma membranes we concluded that the high-affinity Ca^{2+} -ATPase in these membranes is calmodulin-dependent and that the membrane fractions used contained significant amounts of calmodulin, even when low concentrations of EDTA (0.1 mmol l^{-1}) were used throughout the isolation procedure (Flik *et al.* 1984b). In the present study radioimmunoassayable calmodulin was found not only in the cytosol fraction but also in the Ca^{2+} -ATPase-containing plasma membranes, which were isolated in the presence of EDTA (Table 3).

Ca^{2+} -transport

ATP-dependent Ca^{2+} -transport in resealed plasma membrane vesicles of tilapia gill epithelium was assayed in the presence of up to $5 \mu\text{mol l}^{-1}$ Ca^{2+} . An Eadie-Hofstee plot of the results revealed the existence of one affinity site for Ca^{2+} (Fig. 2). The calculated $K_{0.5}$ for Ca^{2+} was $0.13 \mu\text{mol l}^{-1}$ and a V_{max} of $0.7 \text{ nmol Ca}^{2+} \text{ min}^{-1} \text{ mg}^{-1}$ protein at 37°C was found. This V_{max} value is not corrected for the percentage of inside-out vesicles.

Maximum Ca^{2+} -transport rate in whole fish from vesicle transport

In freshwater tilapia, total gill Na^+/K^+ -ATPase activity, calculated as the product of Na^+/K^+ -ATPase specific activity and total protein in H_0 , is directly related to body weight (W) according to: total Na^+/K^+ -ATPase = $4.74W^{1.035} \mu\text{mol } P_i \text{ h}^{-1}$ ($N = 41$; $W: 7\text{--}30 \text{ g}$; $r_0 = 0.792$, $P < 0.001$; Fig. 3). This relationship indicates that the amount of plasma membranes as well as the Na^+/K^+ -ATPase-dependent Na^+ -transport capacity of the gills is directly related to body weight. For a 20-g tilapia, total gill

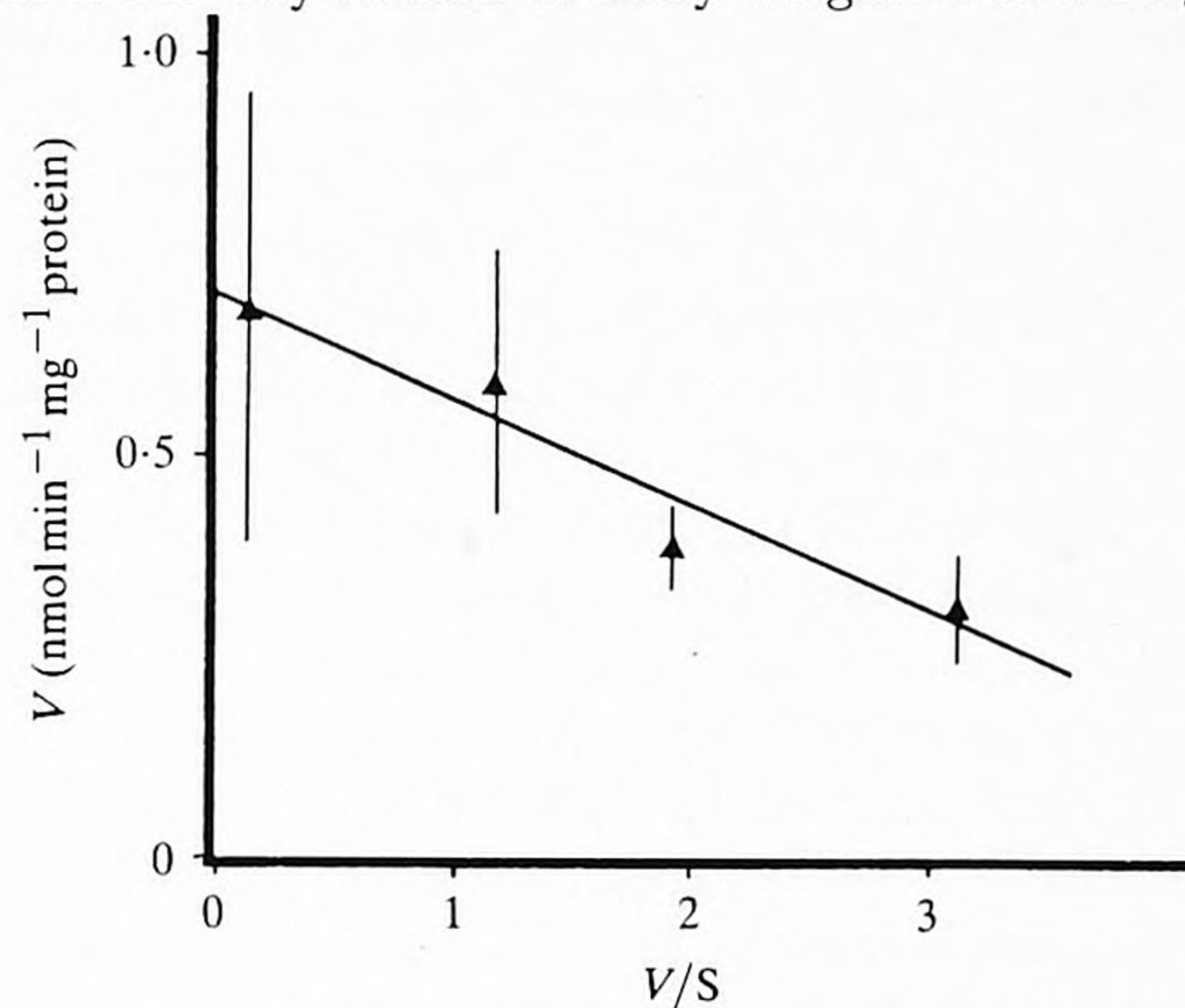


Fig. 2. Eadie-Hofstee plot of Ca^{2+} -stimulated, ATP-dependent Ca^{2+} -transport in tight plasma membrane vesicles. Ca^{2+} -transport activity (V) was determined in 2- to 5-min incubations at 37°C . Ca^{2+} concentrations ranged from 0 to $5 \mu\text{mol l}^{-1}$ (S). Ca^{2+} -transport was expressed in $\text{nmol } Ca^{2+} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$; results were not corrected for percentage of inside-out vesicles.

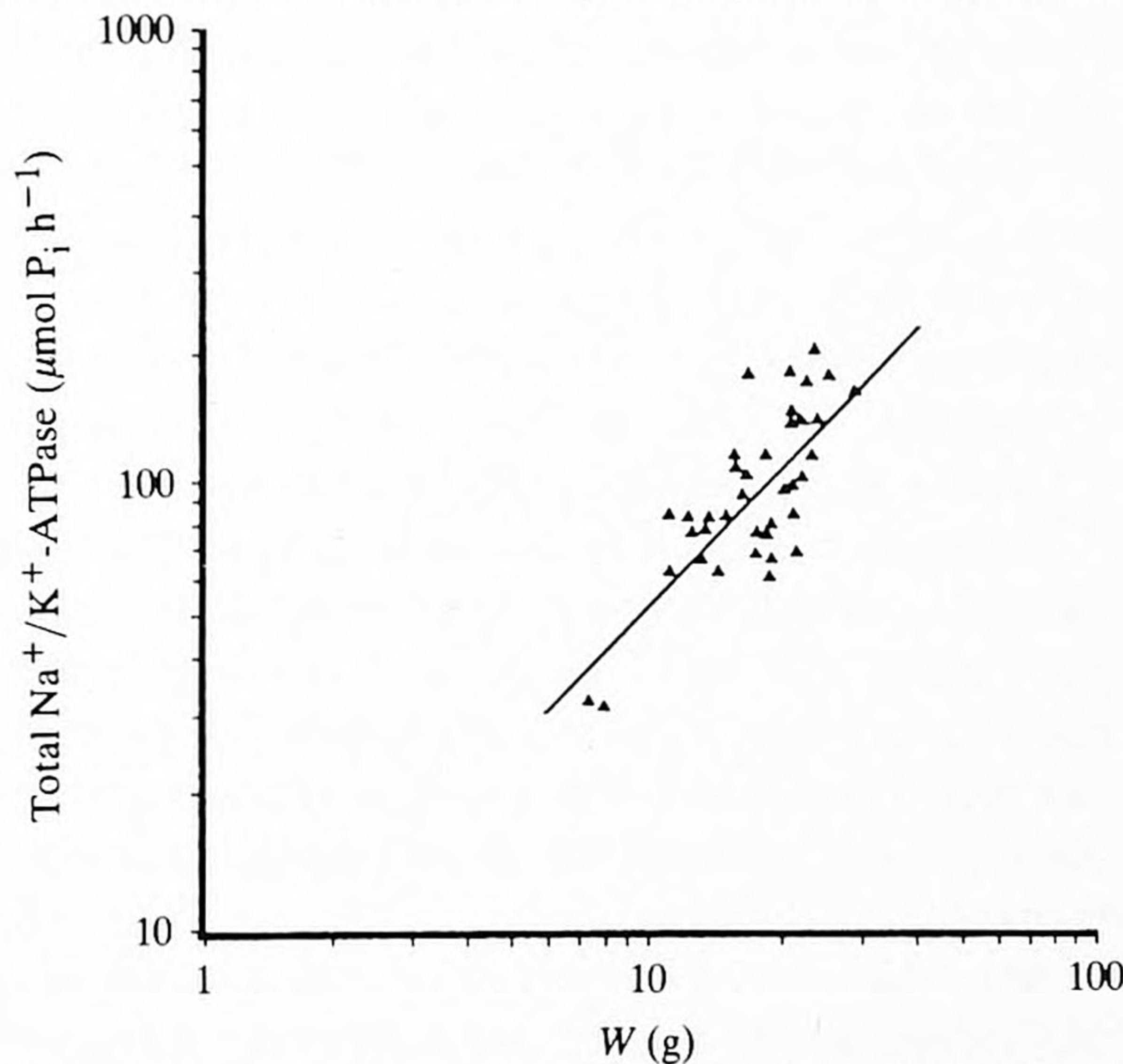


Fig. 3. Full-logarithmic plot of the relationship between total gill Na^+/K^+ -ATPase and body weight (W) of freshwater tilapia. Total Na^+/K^+ -ATPase was calculated as the product of Na^+/K^+ -ATPase specific activity and total protein in H_0 . Results were fitted by linear regression analysis yielding the relationship: total gill Na^+/K^+ -ATPase = $4.74W^{1.035} \mu\text{mol } P_i \text{ h}^{-1}$ (at 37°C).

Na^+/K^+ -ATPase is $105.3 \mu\text{mol P}_i \text{ h}^{-1}$ at 37°C . From an average specific activity of $8.1 \mu\text{mol P}_i \text{ h}^{-1} \text{ mg}^{-1}$ protein in H_0 and 2.82 % recovery for protein in P_3 , it follows that P_3 contains 0.367 mg protein. At a maximum Ca^{2+} -transport rate of $60 \times 0.7 = 42 \text{ nmol Ca}^{2+} \text{ h}^{-1} \text{ mg}^{-1}$ protein, 30 % IOV and 0.367 mg protein in P_3 , the Ca^{2+} -transport capacity of this P_3 -fraction is 51.4 nmol h^{-1} at 37°C . Assuming an activation energy of 57.9 kJ mol^{-1} , as reported for the eel gill Ca^{2+} -ATPase (Flik *et al.* 1984b), the Ca^{2+} -transporting capacity of the P_3 -fraction amounts to 26.2 nmol h^{-1} at 28°C , the temperature at which the tilapia were kept. Further, considering a 4.5 % recovery of Na^+/K^+ -ATPase in P_3 , the total branchial Ca^{2+} -transporting capacity amounts to 580 nmol h^{-1} at 28°C .

Maximum rate of Ca^{2+} -transport ATPase in whole fish

Proceeding from a 20.4 : 1 ratio for Na^+/K^+ -ATPase and Ca^{2+} -ATPase, a total Na^+/K^+ -ATPase activity of $105.3 \mu\text{mol P}_i \text{ h}^{-1}$ for a 20-g fish, a $\text{Ca}^{2+}/\text{ATP}$ stoichiometry of 1 (Sarkadi, 1980) and after correction for temperature, a total Ca^{2+} -transporting capacity of 2633 nmol h^{-1} at 28°C is calculated on the basis of gill total high-affinity Ca^{2+} -ATPase activity.

DISCUSSION

We conclude that there is a high-affinity, calmodulin-dependent Ca^{2+} -ATPase activity in plasma membranes of tilapia branchial epithelium. Moreover, the present data provide evidence for a correlation between this enzyme activity and ATP-driven Ca^{2+} -transport. Kinetic analysis of the Ca^{2+} -transport process by means of an Eadie-Hofstee plot revealed a single class of Ca^{2+} -sites, and half-maximum activation occurred at intracellular Ca^{2+} -concentrations. The data provide the first biochemical evidence for active Ca^{2+} -transport in plasma membranes of teleost branchial epithelium.

Membrane isolation and orientation

To determine high-affinity Ca^{2+} -ATPase activity in isolated plasma membranes by means of colorimetric methods, the availability of highly purified plasma membrane fractions is a prerequisite, for the maximum activity of this enzyme is low (approximately 5 % of the Na^+/K^+ -ATPase activity). Moreover, this activity is clearly dissociated from background ATP-hydrolysis by non-specific phosphatases in these membranes (Flik *et al.* 1984b). The two isolation procedures applied for tilapia gill plasma membranes, methods I and II, yielded membrane fractions that were significantly enriched in Na^+/K^+ -ATPase activity, but only in membrane fractions obtained by method II could successful determination be made of the high-affinity Ca^{2+} -ATPase activity. A major flaw of this isolation procedure is the low recovery of protein in the plasma membrane fraction (P_3'). This allowed only a limited number of determinations on individual fish.

We have previously demonstrated that eel gill plasma membranes contain a similar high-affinity Ca^{2+} -ATPase activity as reported here for tilapia (Flik *et al.* 1983, 1984b). Surprisingly, the same ratio of Na^+/K^+ -ATPase to high-affinity Ca^{2+} -ATPase, namely 20.4 : 1, was observed in the plasma membrane enriched fractions of both species.

The isolation of membranes from tilapia branchial epithelium according to method I resulted in lower recoveries of SDH activity and Na⁺/K⁺-ATPase activity than was the case in the eel gill membrane preparations (Flik *et al.* 1983). In addition, Na⁺/K⁺-ATPase specific activities were about four-fold lower in the tilapia preparations than in the eel preparations. These differences may reflect species-specific differences, or may be attributed to differences in body size or in the composition of the water to which the fish had been adapted. However, the Na⁺/K⁺-ATPase specific activities in tilapia membranes isolated according to method I are similar to those reported for plasma membrane preparations of rat enterocytes (De Jonge *et al.* 1981) or kidney cortex cells (van Heeswijk, Geertsen & Van Os, 1984), preparations that were both shown to possess Ca²⁺-transport activity.

Our results concerning the effects of EDTA and detergent treatment demonstrate that the presence of EDTA during isolation induces a leaky membrane preparation, whereas omission of this chelator during isolation yields a membrane preparation that at least partially consists of tight vesicles. As judged by the relative maximum activities of the Na⁺/K⁺-ATPase, omission of EDTA during isolation does not affect the recovery of this enzyme in the membrane preparation. That resealing occurred of membranes isolated in the absence of EDTA was further substantiated in substrate exclusion tests for acetylcholine esterase and GPDH activities. Average values for composition of the plasma membrane vesicle preparation were: 30 % inside-out, 44 % right-side-out and 26 % leaky vesicles. These figures must be interpreted with care, however, for the 45 % Na⁺/K⁺-ATPase accessibility of this membrane preparation indicates that significant permeation of large molecules (ATP and ouabain) occurred during the 10-min incubation period of the Na⁺/K⁺-ATPase assay. This could imply that the permeability of the membranes to Ca²⁺ changes during incubation. Thus, our calculated 30 % resealed inside-out vesicles may be an overestimate when this value is used to determine the fraction of vesicles in a membrane preparation that shows ATP-driven Ca²⁺-accumulation. Short incubation times, as used in our study, seem to be a prerequisite for the determination of the Ca²⁺-transport capacity of plasma membrane vesicle preparations.

Ca²⁺-stimulated ATPase activity

In our membrane preparations high-affinity Ca²⁺-ATPase activity is strictly associated with Na⁺/K⁺-ATPase activity, which is known to be concentrated in the chloride cells of the gills (Shirai, 1972; Hootman & Philpott, 1979). It is reasonable to suggest then, that the high-affinity Ca²⁺-ATPase is also concentrated in the chloride cells.

A high-affinity and a low-affinity site for Ca²⁺ were deduced for Ca²⁺-stimulated ATP hydrolysis, which corroborates our results on eel gill plasma membranes (Flik *et al.* 1984b). The $K_{0.5}$ value for Ca²⁺ of the low-affinity component of the enzyme activity amounted to 14 $\mu\text{mol l}^{-1}$ Ca²⁺. This makes it unlikely that this component is involved in the extrusion of Ca²⁺ from the cytosol, since the cytosolic Ca²⁺ concentration is generally below 1 $\mu\text{mol l}^{-1}$ Ca²⁺ (Wolf & Brostrom, 1979). In contrast, the $K_{0.5}$ value of 0.063 $\mu\text{mol l}^{-1}$ Ca²⁺ found for the high-affinity component is within the range that may be expected for an intracellularly-stimulated enzyme.

As pointed out previously (Flik *et al.* 1984b) such very high affinities for Ca²⁺ are

an indication that the enzyme is calmodulin-dependent. In eel gill epithelium we have demonstrated that the high-affinity Ca^{2+} -ATPase activity is sensitive to calmodulin antagonists (Flik *et al.* 1984b). In the present study we demonstrated directly the presence of calmodulin in Ca^{2+} -ATPase containing membranes of tilapia gills. A close association of calmodulin with plasma membranes is indicated by the fact that our membranes were isolated in the presence of EDTA. However, whether the Ca^{2+} -ATPase in tilapia gills is also directly associated with calmodulin remains to be demonstrated.

Calmodulin is also abundantly present in cytosol fractions of the branchial epithelium. Since this epithelium is a mixed cell population that includes mucus cells, chloride cells and respiratory cells, we do not know the cellular origin of calmodulin. We have shown earlier that mucus contains calmodulin (Flik *et al.* 1984a). However, mucus and cytosol fractions of branchial epithelium, purified in the same way, differ significantly in their calmodulin content: mucus contains 0.7 mg g^{-1} protein (Flik *et al.* 1984a), cytosol contains 1.98 mg g^{-1} protein. Since mucocytes are almost completely filled up by mucus-containing secretory granules, the calmodulin determined in cytosol in the present study cannot be derived from mucocytes only. The finding that calmodulin is present in Na^+/K^+ -ATPase enriched membrane fractions indicates that the chloride cells also contain an appreciable amount of calmodulin. This conclusion is supported by determinations of calmodulin in preparations of isolated chloride cells: we observed a three-fold higher calmodulin concentration in fractions of isolated chloride cells than in branchial epithelium as a whole (unpublished observations). The level of 0.165 % calmodulin on H_0 -protein basis makes the branchial epithelium a rich source of calmodulin, comparable for example to mammalian testis (Wolf & Brostrom, 1979).

Transepithelial Ca^{2+} -transport in gills

The high-affinity Ca^{2+} -ATPase activity present in tilapia gill membranes is similar to the Ca^{2+} -translocating high-affinity Ca^{2+} -ATPases identified in mammalian erythrocytes and rat enterocytes (Gietzen *et al.* 1981; Ghijsen *et al.* 1982) with respect to its kinetic parameters and calmodulin-dependency. The present demonstration that the gill membrane preparation displaying high-affinity Ca^{2+} -ATPase activity also shows ATP-dependent Ca^{2+} -transport leads to the conclusion that the Ca^{2+} -ATPase activity may be equated with an ATP-dependent Ca^{2+} -transport mechanism. The high affinity for Ca^{2+} of this transport mechanism implies that Ca^{2+} -transport can be stimulated by Ca^{2+} concentrations in the intracellular range, and therefore is involved in Ca^{2+} extrusion from the cytoplasm. The inference that this high-affinity Ca^{2+} -transport mechanism is located in the chloride cells is in line with the conclusion of Payan *et al.* (1981) that these cells provide for virtually all of the branchial Ca^{2+} -transport in fish. We suggest that the high-affinity Ca^{2+} -transport mechanism forms a vital element in the uptake of Ca^{2+} from the ambient water. Since the Ca^{2+} concentration of fresh water is generally 100- to 1000-fold higher than the cytoplasmic Ca^{2+} concentrations reported for eukaryotic cells (Kretsinger, 1980), Ca^{2+} from the water may enter the chloride cells passively, down an electrochemical gradient. The high-affinity Ca^{2+} -ATPase activity may account for the Ca^{2+} -extrusion across the membranes of the tubular system that is continuous with the basolateral cell membranes into the extracellular space and the blood, against a steep Ca^{2+} -gradient (Fig. 4).

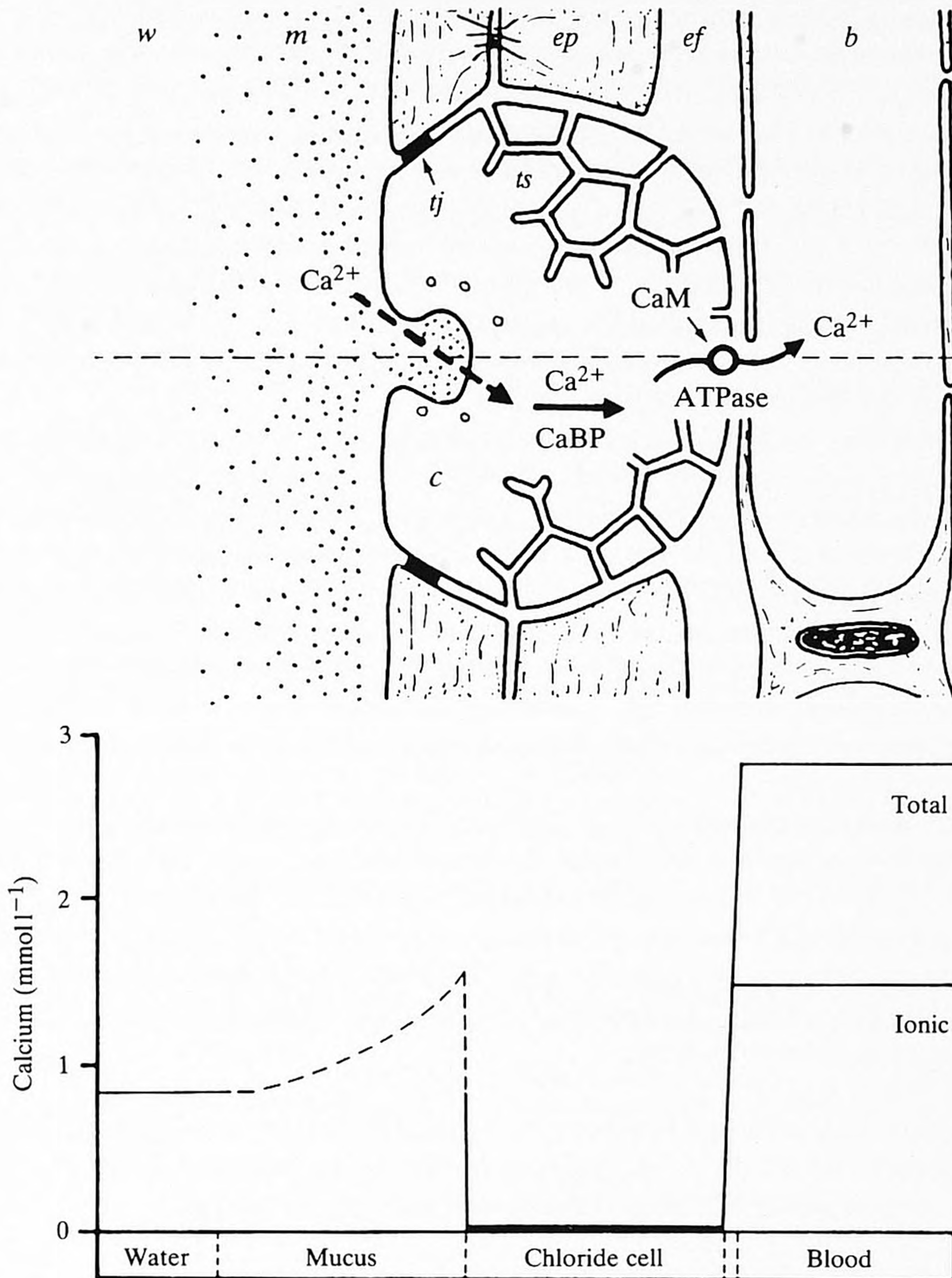


Fig. 4. Model for active Ca^{2+} -uptake in freshwater fish gills. Upper part: diagram of transcellular Ca^{2+} -transport across the branchial epithelium of a freshwater tilapia. Ca^{2+} from the water (*w*) enters the epithelium (*ep*) via chloride cells down an electrochemical gradient. Mucus (*m*) covering the epithelium may steepen the Ca^{2+} -gradient over the apical membrane by concentrating Ca^{2+} topically due to its Ca^{2+} -binding activity as well as by providing an unstirred layer. Ca^{2+} is buffered in the cytosol (*c*) by calcium-binding proteins (CaBP). At the basolateral plasma membranes Ca^{2+} is extruded to the extracellular fluid (*ef*). A Ca^{2+} -transporting enzyme (ATPase), which depends on activation by calmodulin (CaM), provides for the energy to translocate Ca^{2+} against a steep gradient; the basolateral plasma membranes extend to a well-developed system of infoldings referred to as reticular system or tubular system (*ts*); *tj*, tight junctions, *b*, blood. Lower part: Ca^{2+} -concentrations along the dotted line in the upper diagram. Values are as follows: water ($0.8 \text{ mmol l}^{-1} \text{ Ca}^{2+}$, Nijmegen city tap water); mucus (the dotted line indicates that the Ca^{2+} -concentration may increase towards the apical cell membranes); cytosol of chloride cell (approximately $0.1 \mu\text{mol l}^{-1} \text{ Ca}^{2+}$); extracellular fluid and blood (*ef*, *b*; approximately 2.8 mmol l^{-1} total Ca^{2+} and $1.5 \text{ mmol l}^{-1} \text{ Ca}^{2+}$, respectively).

The maximum Ca^{2+} -transport activity of a 20-g tilapia as calculated on the basis of our membrane vesicle studies (580 nmol h^{-1} at 28°C) is approximately 4.5 times lower than the maximum Ca^{2+} -transport activity as calculated on the basis of the total high-affinity Ca^{2+} -ATPase activity present in the branchial apparatus of such a fish. However, since the Ca^{2+} -ATPase determination and the analysis of Ca^{2+} -transport activity required the isolation of membranes by two different procedures, no conclusions concerning the ATP/ Ca^{2+} stoichiometry of the Ca^{2+} -transport can be drawn. Yet, the Ca^{2+} -transport capacity of the gills of a 20-g tilapia, as derived from *in vitro* experiments (580 nmol h^{-1}), compares well with the branchial Ca^{2+} influx rate as determined *in vivo* for a fish of the same size (560 nmol h^{-1} ; Flik *et al.* 1985). Thus it seems justified to ascribe physiological significance to the Ca^{2+} -transport activity observed in our gill membrane preparations.

One could deny such a significance with the argument that a transepithelial potential difference of about 20 mV (inside negative) can also account for the Ca^{2+} influx rates that we observed in intact tilapia. Transepithelial potential differences in this range have been reported for the gills of the brown trout, *Salmo trutta*, for example (McWilliams & Potts, 1978). However, due to the very low cytoplasmic Ca^{2+} concentrations that are reported for cells in general, passive transport must follow the paracellular route, through the tight junctions connecting the epithelial cells (Fig. 4). These junctions, in general, are known to be highly impermeable to Ca^{2+} , and therefore active transcellular Ca^{2+} -transport seems the most likely route for Ca^{2+} uptake from the water.

Further characterization of the Ca^{2+} -transporting mechanism(s) in fish gill epithelium is now being undertaken. In mammals the regulation of the levels of ionized calcium in the cytosol of ion-transporting epithelia may involve, in addition to transport Ca^{2+} -ATPase, a process of coupled $\text{Na}^+/\text{Ca}^{2+}$ exchange across basolateral membranes, energized by a sodium gradient over these membranes (Taylor & Windhager, 1979). The possibility of $\text{Na}^+/\text{Ca}^{2+}$ exchange in branchial Ca^{2+} -transport needs to be considered.

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